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# REAGGREGATION OF INSECT CELLS *IN VITRO*. I. ADHESIVE PROPERTIES OF DISSOCIATED FAT-BODY CELLS FROM DEVELOPING SATURNIID MOTHS

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Cells dissociated from the fat-body of developing saturniid moths undergo rapid reaggregation when cultured in insect blood (Walters and Williams, 1966). The phenomenon as a whole is reminiscent of the self-reconstruction of tissues from the dissociated cells of sponges and vertebrate embryos (Wilson, 1907; Townes and Holtfreter, 1955; Humphreys, 1963; Moscona, 1965). The important difference is that the cells of the dissociated fat-body lack any intrinsic motility or power of locomotion.

By means of time-lapse cinematography, it was possible to show that the dissociated fat-body cells are drawn together by certain motile hemocytes which are regularly present in the hemolymph of developing moths. These "plasmatocytes" were seen to crawl about and frequently adopt a single shape with their elongate processes adhering to two or more fat-body cells or groups of cells which were then pulled together by the active contraction of the plasmatocytes.

In stationary cultures of dissociated fat-body cells, reaggregation was completely blocked when the plasmatocytes were removed or otherwise inactivated. Cultures lacking all hemocytes could be made to reaggregate, however, by subjecting them to gentle agitation. The fat-body cells collided at random and cohered to form large aggregates. This finding strongly implies an intrinsic adhesiveness between fat-body cells. In the experiments reported here, the phenomenon has been further examined in order to identify the factors prerequisite to this mutual adhesion.

## Materials and Methods

### *Experimental animals*

Coccoons of the Polyphemus, Cynthia, and Cecropia silkworms (*Antheraea polyphemus, Samia cynthia,* and *Hyalophora cecorpia;* family Saturniidae) were obtained from dealers and stored in the cold (Polyphemus and Cecropia at 5° C; Cynthia at 8° C). After at least three months of chilling, the pupae were removed from their cocoons and placed at room temperature (22°–25° C). The onset and subsequent stages of adult development were identified by externally visible characters according to the timetables prepared for Polyphemus (Nüesch, 1965; Walters, 1967), Cynthia (Williams, 1968), and Cecropia (Schneiderman and Williams, 1954).

*Dissociated fat-body cells*

Pupae were selected on the third to fifth day after the initiation of adult development. A scalpel incision was made in the mid-abdomen, and by gentle pressure large masses of fat-body were extruded through the wound. The tissue was excised and transferred to centrifuge tubes containing a small volume of physiological saline (Weevers, 1966). Dissociation into single cells was accomplished by drawing the tissue through a Pasteur pipette (bore 1 mm). The resulting suspension was divided among a number of tubes. The cells were collected by centrifugation at the low speed of a clinical centrifuge for one minute. This procedure effectively separated the heavy fat-body cells from hemocytes and debris. The fat-body cells were washed once with Weevers's solution and immediately resuspended as described below.

*Assay for reaggregation*

Hemolymph obtained from diapausing pupae through an incision in the facial region was collected in one or more centrifuge tubes containing crystals of streptomycin and phenylthiourea (Schmidt and Williams, 1953) and then centrifuged at 1500 *g* for 10 minutes to remove all hemocytes.

TABLE 1

*Effect of plasma from different sources on the reaggregation of fat-body cells*

| Source of plasma | Developmental stage of plasma donor | Source of fat-body cells | Reaggregation after 2 hours |
|---|---|---|---|
| Polyphemus | Diapausing pupa | Polyphemus | maximal |
| Polyphemus | Early development | Polyphemus | maximal |
| Polyphemus | Late development | Polyphemus | maximal |
| Polyphemus | Diapausing pupa | Cynthia | maximal |
| Polyphemus | Diapausing pupa | Cecropia | maximal |
| Cynthia | Diapausing pupa | Cynthia | maximal |
| Cynthia | Early development | Cynthia | maximal |
| Cynthia | Diapausing pupa | Polyphemus | maximal |
| Cecropia | Early development | Cecropia | maximal |
| Cecropia | Diapausing pupa | Polyphemus | maximal |

In each series of assays, one aliquot of fat-body cells was resuspended in plasma diluted with an equal part of Weevers's solution; the remaining aliquots were resuspended in various experimental media described below. In each case, sufficient solution was added to give a cell density of *ca.* 100 per $\mu$l, as determined by hemocytometer count.

Reaggregation was assayed by the technique described by Moscona (1961). One to 1.5 ml of cell suspension was placed in a 10-ml Erlenmeyer flask and subjected to gentle swirling at 65 rpm on a rotary shaker (radius of displacement 1 cm; A. H. Thomas Co., Philadelphia). The suspensions were allowed to rotate for 2 to 3 hours, and the degree of agglutination was scored as described in Table II. Except where otherwise noted, the experiments were performed on the cells and plasma of Polyphemus.

TABLE II

*Effect of plasma concentration: degree of reaggregation of fat-body cells in pupal plasma diluted with physiological saline*

(Identical results were obtained for both Polyphemus and Cynthia)

| Amount of plasma in medium | Degree of aggregation after 2–3 hours |
|---|---|
| ≧40% | +++ Maximal; small number of large clumps (average diameter 1 mm), almost no single cells. |
| 20% | ++ Moderate; large to medium clumps, a few single cells. |
| 10% | + Partial; medium to small clumps, half of cells remain single. |
| 5% | (+) Trace; many tiny clusters of only 2–6 cells each, most cells remain single. |
| 2.5% | 0 No apparent agglutination |
| 0% | 0 No apparent agglutination |

## RESULTS

### *Changes in the fat-body during adult development*

The fat-body of diapausing pupae consists of numerous ribbons of branching and anastomosing tissue which surround the viscera and fill most of the abdomen and thorax. On histological examination, individual cells are found to be about 50 $\mu$ in diameter; each is characterized by a central, round nucleus and a cytoplasm charged with large granules which stain metachromatically with toluidine blue. The cells fit intimately together, and the tissue as a whole is surrounded by a thick basement membrane.

Two or three days after the initiation of adult development, the basement membrane disappears. Simultaneously, the strands of tissue fuse into an amorphous mass from which individual cells are easily dissociated by any mechanical stress (*cf.* Ishizaki, 1965; Krishnakumaran, Berry, Oberlander and Schneiderman, 1967).

During the three weeks which the pupal-adult metamorphosis requires at 25° C, the fat-body becomes progressively reduced in volume and restricted to the abdomen. After the tenth day of development, the tissue takes the form of numerous small clusters of cells adhering to other organs and especially to the tracheae. Shortly before the emergence of the adult moth, the stranded morphology of the fat-body is restored; the tissue lies mainly around the abdominal tracheae and is once again invested by a prominent basement membrane. The individual cells are greatly shrunken; they show irregular nuclei and a cytoplasm virtually devoid of granules.

Only during the first third of adult development can the fat-body be readily dissociated. Attempts to dissociate pupal or adult fat body proved unsuccessful even after prolonged immersion in calcium- and magnesium-free saline solutions containing the chelating agent ethylenediaminetetraacetate (EDTA).

### *Reaggregation in suspensions of fat-body cells*

When dispersed fat-body cells were suspended in plasma and subjected to continuous rotary agitation, they promptly began to cohere. As reaggregation com-

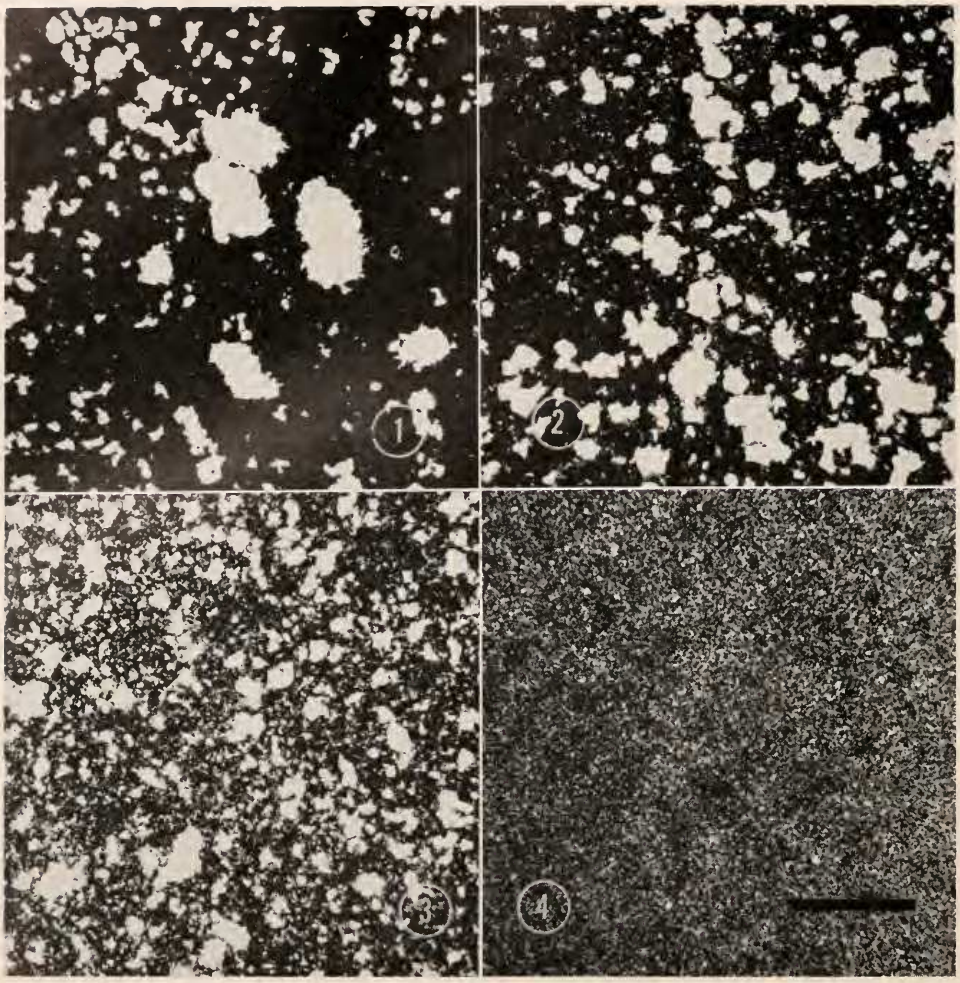


FIGURE 1. Aggregates of fat-body cells which formed after 2 hours of rotation in 40 per cent plasma. (Maximal aggregation.)

FIGURE 2. Same, in 20 per cent plasma. (Moderate aggregation.)

FIGURE 3. Same, in 10 per cent plasma. (Partial aggregation.)

FIGURE 4. Unaggregated cells in Weevers's solution. Black bar represents 4 mm; all Figures at the same magnification.

menced, the suspension became less turbid. Tiny clusters of cells formed and gradually coalesced until maximal aggregation was attained in the course of 2 or 3 hours at room temperature. Fully formed aggregates were compact, spherical, and ranged from a few tenths to more than two millimeters in diameter (Fig. 1).

The aggregates could be again dissociated into single cells by drawing them through a Pasteur pipette. When returned to the rotary shaker, the cells underwent reaggregation as before. Histological examination of fixed and sectioned aggregates revealed intimate cellular contact within each cluster.

*Role of cellular metabolism*

Dissociated fat-body cells were suspended in plasma and placed on a rotary shaker in the cold (2°–3° C). Reaggregation occurred almost as rapidly as at room temperature. In this case, however, the final aggregates were irregular in shape, and individual cells cohered loosely.

A similar result was observed when cell suspensions were treated with $10^{-3}$ M 2,4-dinitrophenol—a concentration which fully inhibited cellular locomotion and reaggregation in stationary cultures (Walters and Williams, 1966). When rotated, the cells agglutinated at the normal rate but formed irregular aggregates. On microscopic examination, these aggregates differed from normal in that the cells were in loose contact and there were many intercellular spaces within the clusters.

Suspensions of cells in plasma were treated with cycloheximide (actidione), an inhibitor of protein synthesis, and incubated for 0.5 hour at room temperature.

TABLE III

*Comparison of the reaggregation of Cynthia cells in plasma derived from Cynthia or from Polyphemus*

| Proportion of plasma in medium | Reaggregation after 2 hours in: | |
|---|---|---|
| | Cynthia plasma | Polyphemus plasma |
| 50% | +++ | ++(+) |
| 20% | ++ | ++ |
| 10% | + | + |
| 0% | 0 | 0 |

The cells were then mechanically dispersed and placed on the rotary shaker. Normal aggregation took place even in the presence of 200 mg/ml of cycloheximide —a concentration four times that which suppresses 90 per cent of amino acid incorporation in the wing tissue of developing moths (M. J. Hughes, Harvard University, personal communication).

*Aggregation-promoting factors in plasma*

As summarized in Table I, fat-body cells were suspended in plasma from either diapausing or developing individuals of the same or different species of saturniids. In all cases, the cells reaggregated in typical fashion.

To learn whether plasma is essential for the cohesion of fat-body cells, samples of plasma were diluted serially with Weevers's solution, and an aliquot of cells was suspended in each dilution. The behavior of the suspensions after two hours of rotation is shown in Table II and in Figures 1–4.

In media containing at least 40 per cent plasma the cells underwent maximal aggregation. When the proportion of plasma was reduced to 20 per cent, the degree of agglutination and the size attained by aggregates markedly decreased. This trend continued until in 2.5 per cent plasma no aggregation took place. Similar results were observed when the experiment was repeated using cells and plasma derived from two different species (Table III).

To study the role of the plasma in cellular adhesiveness, an extract was prepared by stirring cell-free hemolymph into 20 volumes of chilled acetone. Approximately 100 mg of dry, yellow precipitate was obtained from each milliliter of plasma. When this powder was dissolved in 2 or 3 volumes of Weevers' solution, the resulting medium sustained normal reaggregation.

Samples of insect hemolymph were dialyzed against 400 volumes of Weevers' saline for 24 hours at 2°–3° C. When assayed, the dialysate was fully effective in promoting aggregation. Likewise, full activity was retained by a Diaflo ultrafiltration membrane (type UM-1; Amicon Corp., Cambridge, Mass.), suggesting for the active factor a molecular weight greater than 10,000 (manufacturer's specification).

The heat stability of the plasma component required for aggregation was investigated by heating samples of whole plasma in a water bath. After being heated to 50°–55° C for ten minutes, plasma sustained reaggregation only slightly less well than unheated plasma. When plasma was heated to 60°–65° C, a fine precipitate formed which did not dissolve upon cooling; the supernatant was assayed and found to be completely inactive.

To determine whether the factor required for aggregation was proteinaceous, 0.2 per cent crystalline trypsin in Weevers's saline was added to an equal volume of plasma to give a final enzyme concentration of 1 mg/ml. After incubation at 25° C for 12 hours, 10 mg/ml of soybean trypsin inhibitor (SBTI [Sigma Chemical Corp., St. Louis]) was added. To additional samples of plasma, trypsin solution and SBTI were simultaneously added; as a further control, an aliquot of plasma was incubated with SBTI in the absence of enzyme. The trypsin digests and the controls were assayed with freshly dissociated fat-body cells. Both controls showed normal reaggregation, whereas the cells suspended in trypsin digest remained completely dispersed.

*Solubility of the factor*

The foregoing experiments clearly point to one or more plasma proteins as indispensible for the mutual adhesion of fat-body cells. To characterize this factor in further detail, proteins were selectively precipitated from cell-free Polyphemus hemolymph by diluting it with distilled water. After ten-fold dilution, the copious concentrates and the acetone powders were each dissolved in sufficient Weevers' saline. (The material dissolved readily except for a small, gummy fraction which was removed by centrifugation.) This medium, consisting of a saline solution of water-insoluble globulins, was assayed in the usual manner and found to support normal reaggregation.

Two of the aqueous supernatants remaining after precipitation of the globulins were concentrated to the volume of the original plasma by means of the Diaflo ultrafiltration apparatus. Two similiar supernatants were extracted in cold acetone to yield 75 mg of dry precipitate for each milliliter of original plasma. The concentrates and the acetone powders were each dissolved in sufficient Weevers' saline to reconstitute a 50 per cent plasma solution and then assayed. Only a trace of reaggregation could be observed in any of these solutions.

In a similar series of experiments, the globulins were precipitated from Cynthia plasma by twelve-fold dilution with distilled water. The precipitate was dissolved

in Weevers' solution and dialyzed against 300 volumes of the same for 24 hours at 2°–3° C. Meanwhile, the aqueous supernatant was concentrated by dialysis in a bath of Carbowax-6000 (Union Carbide Corp., New York) until the volume of the original plasma was restored; it was then dialyzed against 300 volumes of Weevers' solution. Both the globulin and aqueous fractions were adjusted to a volume equivalent to twice that of the original plasma and assayed. Reaggregation was maximal in the suspensions containing the globulin fraction but almost nil in the aqueous fraction.

Diapausing Polyphemus plasma was dialyzed to remove free amino acids, and its protein content was determined by the method of Lowry, Rosebrough, Farr and Randall (1951). Total protein was found to be 85 mg/ml, of which 15 mg/ml precipitated upon ten-fold dilution with water.

Several vertebrate tissue extracts or purified proteins were assayed for their ability to promote the aggregation of fat-body cells. A crude acetone-precipitate of pig liver (Pentex, Inc., Kankakee, Ill.) was dissolved in Weevers' solution in

Table IV

*Reaggregation of Cynthia fat-body cells suspended in dialyzed plasma*

| Number of experiments | Dialysis bath | Salt added to dialysate | Aggregation after 2 hours |
|---|---|---|---|
| 6 | Weevers' soln. | none | +++ |
| 6 | Ca- & Mg-free soln. | none | 0 or (+) |
| 1 | Ca- & Mg-free | $CaCl_2$ ( 4 mM) | +++ |
| 1 | Ca- & Mg-free | $CaCl_2$ (10 mM) | +++ |
| 1 | Ca- & Mg-free | $MgCl_2$ (18 mM) | +++ |
| 1 | Ca- & Mg-free | $MgCl_2$ (10 mM) | ++(+) |

the amount of 30 mg/ml; fat-body cells suspended in this solution failed to reaggregate. Similar negative results were obtained for 30 mg/ml solutions of bovine serum albumin, porcine alpha or beta globulins (Pentex, Inc.), or rabbit gamma globulin (kindly supplied by Professor A. M. Pappenheimer, Jr.).

*Role of divalent cations*

In stationary cultures of fat-body cells, reaggregation is completely inhibited by the chelating agent EDTA (Walters and Williams, 1966). In the present study, 0.075 *M* EDTA fully inhibited reaggregation in rotating suspensions of fat-body cells. When the concentration of EDTA was reduced to 0.030 *M*, the cells clumped in the normal way. Thus, EDTA is effective only at a concentration equivalent to the combined concentrations of calcium and magnesium ions in saturniid plasma (*cf.* Michejda and Thiers, 1963).

More direct evidence of the participation of calcium and magnesium ions was sought in the following experiments. Precipitated plasma globulins were dissolved in either 0.16 N sodium chloride or a modified Weevers' solution lacking calcium and magnesium. (The latter was prepared by replacing the calcium and magnesium salts with an osmotically equivalent amount of glucose.) Contrary to expectation, reaggregation took place in both media, and only to a slightly lesser degree than in the control medium which contained exogenous calcium and magnesium ions.

This puzzling result suggested that the divalent cations might be bound to the globulins and precipitated with them. Therefore, samples of plasma were subjected to dialysis against 400 volumes of calcium- and magnesium-free saline for 24 hours at 2°–3° C. Following this treatment, the plasma proteins were unable to promote aggregation. The addition of crystals of either calcium chloride or magnesium chloride promptly restored their activity (Table IV).

## Discussion

### *Cellular metabolism and adhesion*

Vertebrate embryonic cells are unable to reaggregate when treated with inhibitors of protein and nucleic acid synthesis (Moscona and Moscona, 1963). The same does not seem to be true of many invertebrate cells. Sea urchin and sponge cells continue to reassemble after the suppression of protein synthesis (Guidice, 1965; Humphreys, 1965). The inhibition of general metabolism prevents reaggregation of invertebrate cells only in situations where cell locomotion is necessary to bring the cells into mutual contact (Guidice, 1965; Walters and Williams, 1966).

The present findings indicate that the adhesiveness of fat-body cells, like that of sea urchin and sponge cells, depends neither on concurrent protein synthesis nor on general metabolism. The fact that loosely structured aggregates formed at low temperatures or in the presence of dinitrophenol suggests that reaggregation normally takes place in two steps: an initial loose cohesion followed by the adoption of a "close packing" arrangement. Perhaps the latter process involves a certain degree of active motility on the part of the cells.

It is possible that the technique of dissociation is responsible for the difference in the ability of vertebrate and invertebrate cells to reaggregate in the absence of protein synthesis. Vertebrate embryonic tissues are commonly dissociated with trypsin, a treatment which may denude the cells of certain proteins essential for adhesion. By contrast, the cells of sponges, sea urchins, and insects are separated by ionic or mechanical means and may retain preformed surface materials destroyed by trypsin.

### *Extrinsic factors determining adhesion*

There are two principal requirements for the successful reaggregation of fat-body cells under the experimental conditions described here. First, the medium must contain divalent cations. This requirement has also been recognized in other reaggregating systems (Galtsoff, 1925; Moscona and Moscona, 1952; Humphreys, 1963). Indeed, treatment with chelating agents or immersion in calcium- and magnesium-free media have often been used to dissociate embryonic or even adult tissues (Anderson, 1953; Guidice, 1962; Jones and Elsdale, 1963). The role of calcium has received primary attention. In the case of sponges, however, calcium and magnesium salts have been found interchangeable (Humphreys, 1963), and the same is true of dissociated fat-body cells (Table IV).

In a study of the ionic composition of silkworm plasma, Michejda and Thiers (1963) found unusually high concentrations of divalent cations, especially magnesium. Much lower concentrations are known to be effective in physiological solutions (Weevers, 1966), implying that the ions are extensively bound to other

molecules. The presence of divalent cations in the precipitated globulins, as demonstrated in the present study, confirms that significant amounts of these ions are normally bound to plasma proteins.

A second requirement for the reaggregation of fat-body cells is that the medium contain a water-insoluble proteinaceous material present in insect plasma at all stages of development. The requirement is specific in that it was not possible to substitute non-insect proteins (*viz.*, vertebrate liver extract, serum albumin, or alpha, beta, or gamma globulins). It is not species-specific, however, since the hemolymph of different genera of silkworms was fully effective when interchanged (Tables I and III).

Various proteins, exogenous or produced by the cells themselves, have also been implicated in other reaggregating systems. Certain sponge cells secrete a highly specific glycoprotein which is essential to their mutual cohesion (Humphreys, 1967). The dissociated cells of vertebrate embryos reaggregate only in complex media. Evidently, a number of proteinaceous factors must be present, many of which appear to be the product of cellular metabolism (Moscona, 1965). A factor in the supernatant of avian tissue cultures has been recently shown specifically to enhance the aggregation of dissociated cells from the same kind of tissue (Lilien, 1968; Kuroda, 1968). These findings have been interpreted as evidence for specific macromolecules which cement cells together (Moscona, 1968). Other workers question the existence of such binding substances and consider the soluble proteins to serve an ancillary function in adhesion (Steinberg, 1964; Curtis and Greaves, 1965; Jones, 1966; Curtis, 1967).

The function of the divalent cations has also been subject to various interpretations. Among the postulated effects have been the following: (1) formation of intercellular bridges (Steinberg, 1958); (2) stabilization of extracellular cementing material (Chambers and Chambers, 1961); (3) reduction of electrostatic repulsion between apposing membranes (Curtis, 1962); (4) participation in the enzymatic activity of adhesive sites on the cell surface (Jones, 1966). In the case of fat-body cells, present evidence does not allow us to decide among these possibilities. Despite this fact, it is amply clear that cellular adhesiveness requires divalent cations as well as one or more plasma proteins. Evidently, these factors are intimately related, since reassociation of the fat-body cells was sustained by the calcium and magnesium precipitated with the active proteinaceous material.

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## Summary

1. Cells dissociated from the fat-body of developing saturniid moths underwent rapid reaggregation when subjected to rotary agitation in blood plasma.

2. The rate and degree of aggregation was unaffected by low temperature or the presence of inhibitors such as dinitrophenol or cycloheximide.

3. Normal aggregation also occurred when plasma from different developmental stages or different silkworm genera was substituted.

4. When the proportion of plasma in the medium was reduced to less than 40 per cent, the degree of aggregation as measured by the size of aggregates progressively declined. No aggregation took place in physiological saline.

5. Cell adhesion was found to depend on a plasma factor which was non-dialyzable, heat-labile, inactivated by trypsin and insoluble in distilled water.

6. Vertebrate proteins (liver extract, serum albumin, or alpha, beta, or gamma globulins) could not be substituted for the plasma factor.

7. Cell adhesion also required the presence of either calcium or magnesium ions. These ions precipitated with the active proteinaceous material in amounts sufficient for reaggregation.

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